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ORIGINAL ARTICLE

Protein kinase C-θ regulates KIT expression and proliferation in gastrointestinal stromal tumors

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Oncogenic KIT or PDGFRA receptor tyrosine kinase mutations are compelling therapeutic targets in gastrointestinal stromal tumors (GISTs), and the KIT/PDGFRA kinase inhibitor, imatinib, is standard of care for patients with metastatic GIST. However, most of these patients eventually develop clinical resistance to imatinib and other KIT/PDGFRA kinase inhibitors and there is an urgent need to identify novel therapeutic strategies. We reported previously that protein kinase C-θ (PKCθ) is activated in GIST, irrespective of KIT or PDGFRA mutational status, and is expressed at levels unprecedented in other mesenchymal tumors, therefore serving as a diagnostic marker of GIST. Herein, we characterize biological functions of PKCθ in imatinib-sensitive and imatinib-resistant GISTs, showing that lentivirus-mediated PKCθ knockdown is accompanied by inhibition of KIT expression in three KIT+/PKCθ+ GIST cell lines, but not in a comparator KIT+/PKCθ− Ewing's sarcoma cell line. PKCθ knockdown in the KIT+ GISTs was associated with inhibition of the phosphatidylinositol-3-kinase/AKT signaling pathway, upregulation of the cyclin-dependent kinase inhibitors p21 and p27, antiproliferative effects due to G_1 arrest and induction of apoptosis, comparable to the effects seen after direct knockdown of KIT expression by *KIT* short-hairpin RNA. These novel findings highlight that PKCθ warrants clinical evaluation as a potential therapeutic target in GISTs, including those cases containing mutations that confer resistance to KIT/PDGFRA kinase inhibitors.
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Introduction

Tumorigenesis is a complex, multistep process, and oncogenic tyrosine kinase proteins are important in development of many human cancers (Gschwind *et al*., 2004). Ligand-mediated receptor tyrosine kinase (RTK) activation can regulate proliferation, survival, migration and invasiveness in non-neoplastic cells, whereas oncogenic RTK mutations can induce constitutive kinase activation, and thereby enhance proliferation and survival in cancer cells (Drevs *et al*., 2003; Medinger and Drevs, 2005). Mutant RTKs are useful therapeutic targets, as shown by the clinical successes of kinase inhibitor therapies in chronic myeloproliferative disorders, metastatic breast cancer, gastrointestinal stromal tumor (GIST) and non-small cell lung cancer (Gschwind *et al*., 2004; Paez *et al*., 2004). GISTs arise from transformed progenitor cells committed to differentiation along the interstitial cell of Cajal lineage and are the most common mesenchymal tumors of the gastrointestinal tract (Fletcher *et al*., 2002). GISTs are heterogeneous histologically, including spindle cell (70%), epithelioid cell (20%) and mixed types (10%) (Corless *et al*., 2004). Most GISTs contain oncogenic gain-of-function RTK mutations, involving *KIT* (~85%) or *PDGFRA* (~5%), which are accompanied by strong expression of the protein products of these oncogenes (Hirota *et al*., 1998; Heinrich *et al*., 2003; Corless *et al*., 2004). KIT oncoproteins remain crucial to the proliferation and survival of GIST cells in patients with metastatic GIST, as evidenced by the clinical successes of KIT kinase inhibition by imatinib (Gleevec) and sunitinib (Sutent), *in vitro* and in the clinic (Tuveson *et al*., 2001; Demetri *et al*., 2002). Indeed, imatinib therapy has rapidly become the standard of care in patients with metastatic GIST (Blay *et al*., 2005).

PKCθ is a member of the novel family of protein kinase C proteins, and—unlike the conventional protein kinase C proteins—shows a narrow range of expression in human cells. PKCθ expression was described initially in T and myogenic cells (Baier *et al*., 1993; Chang *et al*., 1993), but can be expressed at even higher levels in GIST (Allander *et al*., 2001; Nielsen *et al*., 2002; Duensing *et al*., 2004a; Medeiros *et al*., 2004). PKCθ is a calcium-independent but phospholipid-dependent

dual-specificity kinase, which is activated by the second messenger diacylglycerol (Teixeira *et al*., 2003). PKCθ biologic roles have been evaluated extensively in T cells, where PKCθ regulates interleukin-2 production and CD25 expression by activating nuclear factor-κB (NFκB) and activator protein-1 (Sun *et al*., 2000; Bi *et al*., 2001). PKCθ:NFκB interactions can be bidirectional, as in the case of T-cell receptor (TCR)-induced NFκB activation, regulated by 3-phosphoinositide-dependent kinase 1, which can activate PKCθ, resulting in PKCθ recruitment to lipid rafts (Lee *et al*., 2005). TCR pathways also regulate PKCθ tyrosine phosphorylation, which is mediated by SRC family protein tyrosine kinases such as LCK (Liu *et al*., 2000). In addition, PKCθ can regulate gene expression, functioning as a positive modulator of retinoid X receptor-responsive element (RXRE)-dependent transcription during T-cell activation (Ishaq *et al*., 2002). PKCθ also has biologic roles outside T-cell activation, including phosphorylation-mediated regulation of cytoskeletal assembly and barrier permeability in intestinal monolayers (Banan *et al*., 2004, 2005). Equally compelling roles for PKCθ are likely in GIST, given the strong expression and activation of PKCθ in these tumors (Blay *et al*., 2004; Duensing *et al*., 2004a; Motegi *et al*., 2005). Although preliminary studies were consistent with a PKCθ prooncogenic role in GIST (Duensing *et al*., 2004a), conventional PKCs can inhibit ligand-mediated KIT activation, in a negative feedback loop, by phosphorylating KIT on S741 and S746 (Blume-Jensen *et al*., 1993, 1994, 1995). Therefore, it is unclear whether PKCθ has positive or negative regulatory roles, or both, in KIT-mutant GISTs.

Although KIT inhibition by imatinib represents a major therapeutic advance for patients with inoperable GIST, most patients eventually experience clinical progression due to the emergence and outgrowth of multiple imatinib-resistant GIST clones (Heinrich *et al*., 2006). These imatinib-resistant clones often contain secondary mutations in the KIT kinase domain (Heinrich *et al*., 2006). Novel multi-targeted kinase inhibitors such as sunitinib show activity in some patients with imatinib-resistant GIST, but are not uniformly effective, and it is therefore important to identify alternative signaling targets for therapies in GIST. PKCθ is an attractive therapeutic target in GIST because it is expressed in few normal cell types. Therefore, highly selective PKCθ inhibitor therapies might be accompanied by minimal toxicities in GIST patients.

In the present study, PKCθ and KIT protein expression were stably silenced by lentiviral *PKCθ* and *KIT* short-hairpin RNA (shRNA) constructs in a KIT+/PKCθ+ imatinib-sensitive GIST cell line (GIST882), two KIT+/PKCθ+ imatinib-resistant GIST cell lines (GIST48 and GIST430) and a control KIT+/PKCθ− Ewing's sarcoma cell line (EWS502). Our findings show that PKCθ regulates KIT expression, cell proliferation and cell survival in GISTs, suggesting that PKCθ warrants evaluation as a therapeutic target in GISTs, including those with resistance to KIT kinase inhibitors such as imatinib.

Results

PKCθ is expressed and strongly phosphorylated in KIT+ GIST cell lines

PKCθ expression and phosphorylation were evaluated in three GIST cell lines (GIST882, GIST48 and GIST430) that express KIT oncoproteins strongly, and are therefore referred to as 'KIT positive', and in two GIST cell lines (GIST62 and GIST522) that lack KIT expression, both of which were established from KIT-positive GISTs, and contain genomic *KIT* mutations. (Figure 1). PKCθ expression—and phosphorylation at T538 and S676, which are required for PKCθ activation—were comparable, or greater, in the KIT-positive GIST lines than in Jurkat T-cell leukemia, which has previously provided a benchmark for high levels of PKCθ expression (Figure 1). By contrast, the two KIT-negative GIST lines showed little or no demonstrable PKCθ expression, and PKCθ S676 phosphorylation was nearly undetectable (Figure 1). In general, PKCθ phosphorylation in the various GIST cell lines paralleled the expression of total PKCθ (Figure 1).

PKCθ regulates KIT oncoprotein expression

To evaluate PKCθ roles in imatinib-sensitive and imatinib -resistant GISTs, *PKCθ* gene expression was stably silenced by lentivirus-mediated shRNA in GIST882, GIST48 and GIST430, and immunoblotting studies were then performed at 96 h post-infection. *KIT* gene silencing, also using lentiviral shRNA constructs, was evaluated in parallel studies (Figure 2a; expression quantitations provided in Supplementary Figure 1).

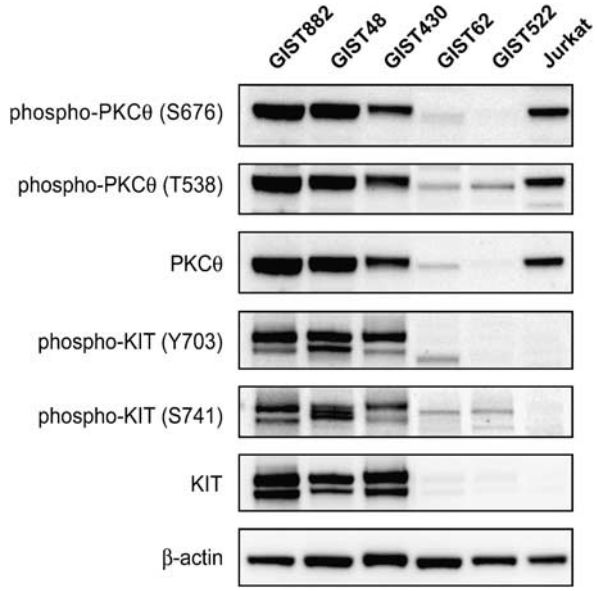


Figure 1 Immunoblotting evaluations of gastrointestinal stromal tumor (GIST) cell lines show strong protein kinase C-θ (PKCθ) activation and expression in KIT-positive lines (GIST882, GIST430 and GIST48) but not in KIT-negative lines (GIST62 and GIST522). The Jurkat T-cell acute lymphoblastic leukemia provides a positive control for PKCθ activation and expression.

The shRNA transductions resulted in greater than 60% inhibition of their intended targets. PKCθ and KIT phosphorylation were also inhibited, commensurate with the reductions in total protein expression, after the shRNA interventions (Figure 2a). KIT oncoprotein expression and phosphorylation, evaluated 96 h after *PKCθ* shRNA infection, were substantially inhibited in the GIST48 and GIST430 imatinib-resistant GIST lines, but not in GIST882 (Figure 2a). However, *KIT* shRNA knockdown did not inhibit PKCθ expression in any of the GIST lines. All lentiviral experimental results were corroborated by at least two separate infections of the various GIST cell lines, and using at least one additional *KIT* and *PKCθ* shRNA sequence (data not shown).

To confirm that the *PKCθ* shRNA construct was specific for its intended target, and not binding directly to *KIT* mRNA, control transductions were performed in the KIT + /PKCθ− Ewing's sarcoma cell line, EWS502. EWS502 cells express wild-type KIT protein, but do not express PKCθ (Figure 2b). EWS502 KIT expression was inhibited at 96 h after infection with *KIT* lentiviral shRNA, but was unaffected after infection with lentiviral *PKCθ* shRNA (Figure 2b). Similarly, reduction in KIT expression was seen in EWS502 cells stably expressing *KIT* shRNA, but not in EWS502 cells stably expressing *PKCθ* shRNA, after 10 and 20 days of selection with 2.5 μg/ml puromycin (Figure 2b).

Effects of PKCθ *and* KIT *shRNA knockdown on GIST signaling pathways*

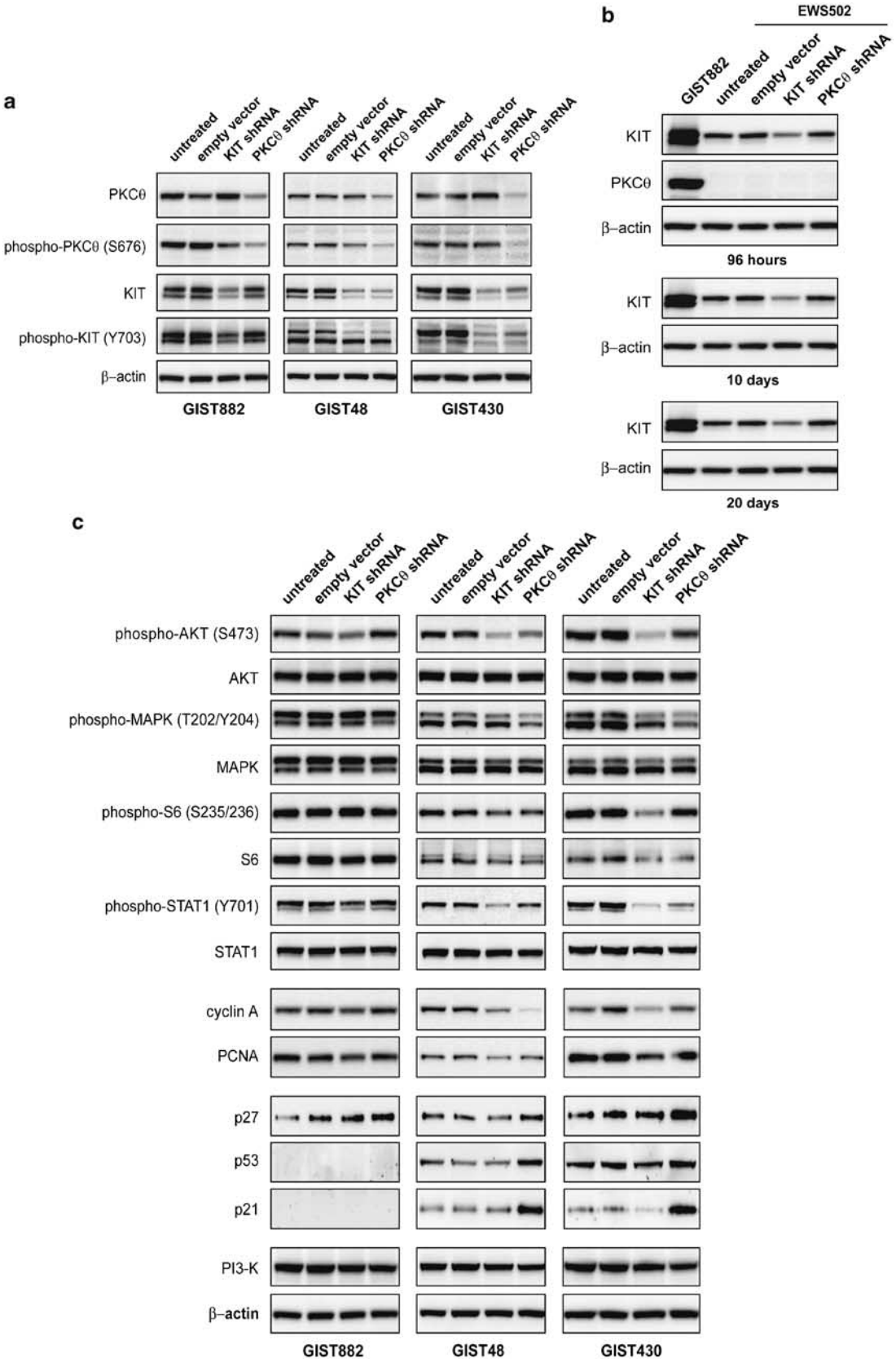
Alterations in proliferation and survival signaling pathways were determined by immunoblotting in the GIST882, GIST48 and GIST430 cell lines after shRNA-mediated inhibition of KIT and PKCθ expression (Figure 2c; expression quantitations provided in Supplementary Figure 2). These studies evaluated phosphorylation of AKT and mitogen-activated protein kinase (MAPK), among other signaling intermediates that have been shown to be KIT dependent in GISTs (Corless *et al*., 2004; Duensing *et al*., 2004b; Bauer *et al*., 2007). Evaluations at 4 days after lentiviral shRNA infection showed that KIT silencing resulted in dramatic inactivation of AKT and S6 in the imatinib-resistant GIST48 and GIST430 cells, whereas MAPK was inactivated partially only in GIST430 (Figure 2c). PKCθ knockdown resulted in at least 50% MAPK inactivation in both GIST48 and GIST430 cells, but not in GIST882 (Figure 2c). Likewise, PKCθ knockdown partially inactivated AKT, S6 and STAT1 in GIST48 and GIST430, but not in GIST882 (Figure 2c). Evaluations, performed in puromycin-selected GIST882 cells with stable *KIT* and *PKCθ* shRNA expression, at 10 and 20 days after lentiviral infection, confirmed that AKT, but not MAPK, was inactivated after KIT silencing (Figure 3a; expression quantitations provided in Supplementary Figure 3). PKCθ knockdown, after 10 days and particularly after 20 days of antibiotic selection, resulted in substantial inactivation of AKT, and marked reduction in expression of total and phosphorylated KIT (Figure 3a). Notably, these studies also reveal that substantial PDGFRA expression is substantially repressed after PKCθ knockdown, but is unaffected after KIT knockdown (Supplementary Figure 3).

Effects of PKCθ *and* KIT *shRNA knockdown on GIST proliferation*

KIT shRNA knockdown inhibited expression of the cyclin A and proliferating cell nuclear antigen (PCNA) proliferation markers in the imatinib-resistant GIST48 and GIST430 cell lines (Figure 2c). *PKCθ* shRNA knockdown also inhibited cyclin A expression in GIST48 and GIST430, and inhibited PCNA expression in GIST48 (Figure 2c). Cyclin A and PCNA expression were unaffected by *PKCθ* shRNA knockdown in GIST882 at 96 h (Figure 2c), and were not substantially downregulated in these cells after 10 and 20 day puromycin selections for stable expression of *KIT* and *PKCθ* shRNAs (Figure 3a). However, GIST882 cell growth was reduced dramatically, when evaluated after 10 and 20 days of *KIT* and *PKCθ* shRNA expression (Figure 3b).

Cellular proliferation was evaluated by an ATP-based cell viability assay (CellTiter-Glo) in GIST882, GIST48 and GIST430 after transduction with *KIT* or *PKCθ* shRNAs. Antiproliferative effects were greater in GIST882 and GIST48 cells after treatment with *PKCθ* shRNA than after treatment with *KIT* shRNA. Antiproliferative effects in GIST430 cells were comparable after treatment with *PKCθ* and *KIT* shRNAs (Figures 2c

Figure 2 (**a**) Immunoblotting evaluations of KIT and protein kinase C-θ (PKCθ) expression and activation, in KIT-positive gastrointestinal stromal tumor (GIST) cell lines (GIST882, GIST430 and GIST48) at 96 h after infection by lentiviral *KIT* or *PKCθ* short-hairpin RNA (shRNA) constructs. *KIT* shRNA infection inhibited KIT expression and activation in each cell line. *PKCθ* shRNA inhibition inhibited PKCθ expression and activation in each cell line, and also inhibited KIT expression and activation in the imatinib-resistant GIST48 and GIST430 cell lines. Control lanes, for each cell line, include uninfected cells (untreated lane) and cells infected with empty lentiviral vector. (**b**) Comparison immunoblotting evaluations of KIT and PKCθ expression, after infection of KIT-positive Ewing's sarcoma (EWS502) cells with lentiviral *KIT* or *PKCθ* shRNA constructs. These studies show that *PKCθ* shRNA treatment does not inhibit KIT expression, in the absence of PKCθ expression, indicating that *PKCθ* shRNA-mediated KIT inhibition in GIST (**a**) does not result from nonspecific interactions between the *PKCθ* shRNA and *KIT* mRNA. The GIST882 cells provide a positive control for KIT and PKCθ expression. EWS502 no-treatment controls include uninfected cells (untreated lane) and EWS502 infected with empty lentiviral vector. (**c**) Immunoblotting evaluations of KIT-positive GIST cell lines (GIST882, GIST430 and GIST48) at 96 h after infection by lentiviral *KIT* or *PKCθ* shRNA constructs. The immunoblotting assays evaluated affects of KIT and PKCθ knockdown on signaling intermediates (AKT, mitogen-activated protein kinase (MAPK) p42/44, S6, STAT1), proliferation markers (cyclin A and proliferating cell nuclear antigen, PCNA) and cell-cycle checkpoint proteins (p27, p21 and p53). The empty vector lane is a parallel control. The phosphatidylinositol-3-kinase (PI3-K) and actin immunostains show equivalence of lane loading. Control lanes, for each cell line, include uninfected cells (untreated lane) and cells infected with empty lentiviral vector.


a
untreated
empty vector
KIT shRNA
PKCθ shRNA
PKCθ
phospho-PKCθ (S676)
KIT
phospho-KIT (Y703)
β–actin
GIST882
GIST48
GIST430
b
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GIST882
untreated
empty vector
KIT shRNA
PKCθ shRNA
KIT
PKCθ
β–actin
96 hours
10 days
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c
phospho-AKT (S473)
AKT
phospho-MAPK (T202/Y204)
MAPK
phospho-S6 (S235/236)
S6
phospho-STAT1 (Y701)
STAT1
cyclin A
PCNA
p27
p53
p21
PI3-K
β–actin

and 4a). All proliferation studies were corroborated by at least two independent shRNA transductions.

Proliferation-related consequences of KIT and PKCθ knockdown were also determined by assessing immunoblot expression of p53 and the p21 and p27 cyclin-dependent kinase (CDK) inhibitors (Figure 2c). GIST48 and GIST430 cells demonstrated upregulation of p53, p21 and p27 expression after PKCθ knockdown, but not after KIT knockdown. Likewise, p27 expression was upregulated in GIST882 cells after PKCθ knockdown, whereas p21 and p53 were not demonstrably expressed in these cells (Figure 2c).

Apoptosis and cell-cycle analysis after PKCθ *and* KIT *gene knockdown*

Apoptosis was evaluated by assaying functional caspase 3/7 activation in GIST882 and GIST48 after transduction with *KIT* or *PKCθ* shRNAs. Apoptosis was more strongly induced in these GIST cells after treatment with *PKCθ* shRNA, compared to treatment with *KIT* shRNA (Figure 4b). Cell-cycle analysis in GIST48 showed a G_1 block after KIT and PKCθ silencing with an increase in the $G_{1/0}$ peak from 83.2% after empty vector transduction to 89.9 and 95.2% after transduction with *KIT* and *PKCθ* shRNAs, respectively (Figure 4c). This was accompanied by a decrease in the S-phase population from 9% with the empty vector control to 5.6 and 2.5% with *KIT* and *PKCθ* shRNAs, respectively (Figure 4c). In addition, the pre-G_1 nuclear fragmentation peak increased from 2% with empty vector control to 7 and 17% with *KIT* and *PKCθ* shRNAs, respectively (Figure 4c). Cell-cycle analysis in GIST882 showed a G_1 block after KIT silencing with an increase in the $G_{1/0}$ peak from 67.9% after empty vector transduction to 72.2% after transduction with *KIT* shRNA (Figure 4c). The pre-G_1 nuclear fragmentation peak in GIST882 increased from 1% with empty vector control to 1.4 and 6.1% with *KIT* and *PKCθ* shRNAs, respectively (Figure 4c).

Discussion

Most GISTs contain oncogenic gain-of-function mutations in the KIT or PDGFRA RTK proteins. These mutations can be early—and even initiating—transforming events (Corless *et al*., 2004). Therefore, KIT/PDGFRA inhibition by small-molecule kinase inhibitors such as imatinib mesylate or sunitinib malate has become the mainstay of treatment in patients with inoperable GIST. At the same time, studies of human GIST cell lines and transgenic mouse models have enabled substantial advances in understanding the central roles of KIT/PDGFRA signaling pathways in GIST cell proliferation and survival (Demetri *et al*., 2002; Heinrich *et al*., 2003; Duensing *et al*., 2004b; Corless *et al*., 2005; Rossi *et al*., 2006; Bauer *et al*., 2007; Zhu *et al*., 2007).

Various studies have shown that PKCθ is expressed strongly in GISTs, but not in other sarcoma histotypes (Blay *et al*., 2004; Duensing *et al*., 2004a; Motegi *et al*., 2005). These studies established PKCθ as a diagnostic marker of GIST, but did not illuminate biological functions of PKCθ. In the present work, we find strong PKCθ expression in each of three KIT-positive GIST cell lines, whereas PKCθ expression was weak-to-undetectable in two KIT-negative GIST lines (Figure 1). These observations suggest that loss of PKCθ expression could be responsible for inhibition of KIT expression in the KIT-negative GIST lines, both of which were established from KIT-positive GISTs, and contain genomic *KIT* mutations. By contrast, we have reported that PKCθ is expressed in untreated KIT-negative GISTs that lack *KIT* mutations but often contain oncogenic *PDGFRA* mutations as an alternate RTK activation mechanism. Although PKCθ is therefore not the sole determinant of KIT expression in GISTs, our data support a key PKCθ role in enabling KIT oncogenic function in GISTs. It is also possible that KIT oncogenic signaling contributes to PKCθ activation in GISTs. KIT activation is known to result in binding and activation of phosphatidylinositol-3-kinase (PI3-K) and phospholipase C-γ, which regulate, respectively, membrane translocation of PKCθ and synthesis of the PKCθ cofactor diacylglycerol (Villalba *et al*., 2002; Altman and Villalba, 2003). Further, we have shown that KIT oncoproteins complex with and tyrosine phosphorylate PKCθ in GISTs, and might thereby directly activate PKCθ (Zhu *et al*., 2007). These observations suggest that KIT and PKCθ participate in a positive feedback loop in GIST.

PKCθ-mediated regulation of KIT oncoprotein expression was demonstrated in each of three KIT-positive GIST cell lines, including the imatinib-sensitive line, GIST882, and the imatinib-resistant lines, GIST48 and GIST430. However, KIT oncoprotein expression was inhibited within 4 days of PKCθ shRNA transduction in GIST48 and GIST430, whereas similar effects were seen only at 10–20 days after PKCθ shRNA transduction in GIST882 (Figures 2a and 3a). One explanation for these observations is that the KIT oncoprotein in GIST882 (K642E mutant) might be more stable after PKCθ knockdown compared to the hyperactivated (Bauer *et al*., 2007) double-mutant KIT oncoproteins in GIST48 and GIST430. Alternately, other biological variables in these unique GIST models might account for the different times of onset for KIT oncoprotein inhibition, after PKCθ knockdown. The specificity of these findings was substantiated in an Ewing's sarcoma cell line (EWS502) that expresses KIT but not PKCθ, and in which PKCθ shRNA infection had no impact on KIT expression (Figure 2b). These studies confirm that perturbations of KIT expression after PKCθ shRNA infection in GIST lines were indeed mediated by the observed PKCθ silencing in those lines. Our preliminary studies (data not shown) suggest that PKCθ modulation of KIT expression in GIST involves regulation of KIT transcription. However, further studies are needed to determine whether KIT expression is regulated by PKCθ biochemical activation or by PKCθ scaffolding functions, but—either way—our findings show that

Figure 3 (**a**) Immunoblotting evaluations of GIST882 cells, at 10 and 20 days after infection by lentiviral *KIT* or protein kinase C-θ (*PKCθ*) short-hairpin RNA (shRNA) constructs. *KIT* shRNA resulted in decreased expression of KIT, phospho-AKT, phospho-S6 and the proliferation markers cyclin A and proliferating cell nuclear antigen (PCNA). *PKCθ* shRNA resulted in decreased expression of PKCθ, KIT, phospho-AKT and cyclin A. The phosphatidylinositol-3-kinase (PI3-K) and β-actin immunostains show equivalence of lane loading. (**b**) GIST882 cell culture appearance, when evaluated at 10 and 20 days after infection by lentiviral *KIT* or *PKCθ* shRNA constructs, showing growth inhibition compared to control GIST882 cultures infected with lentiviral empty constructs.

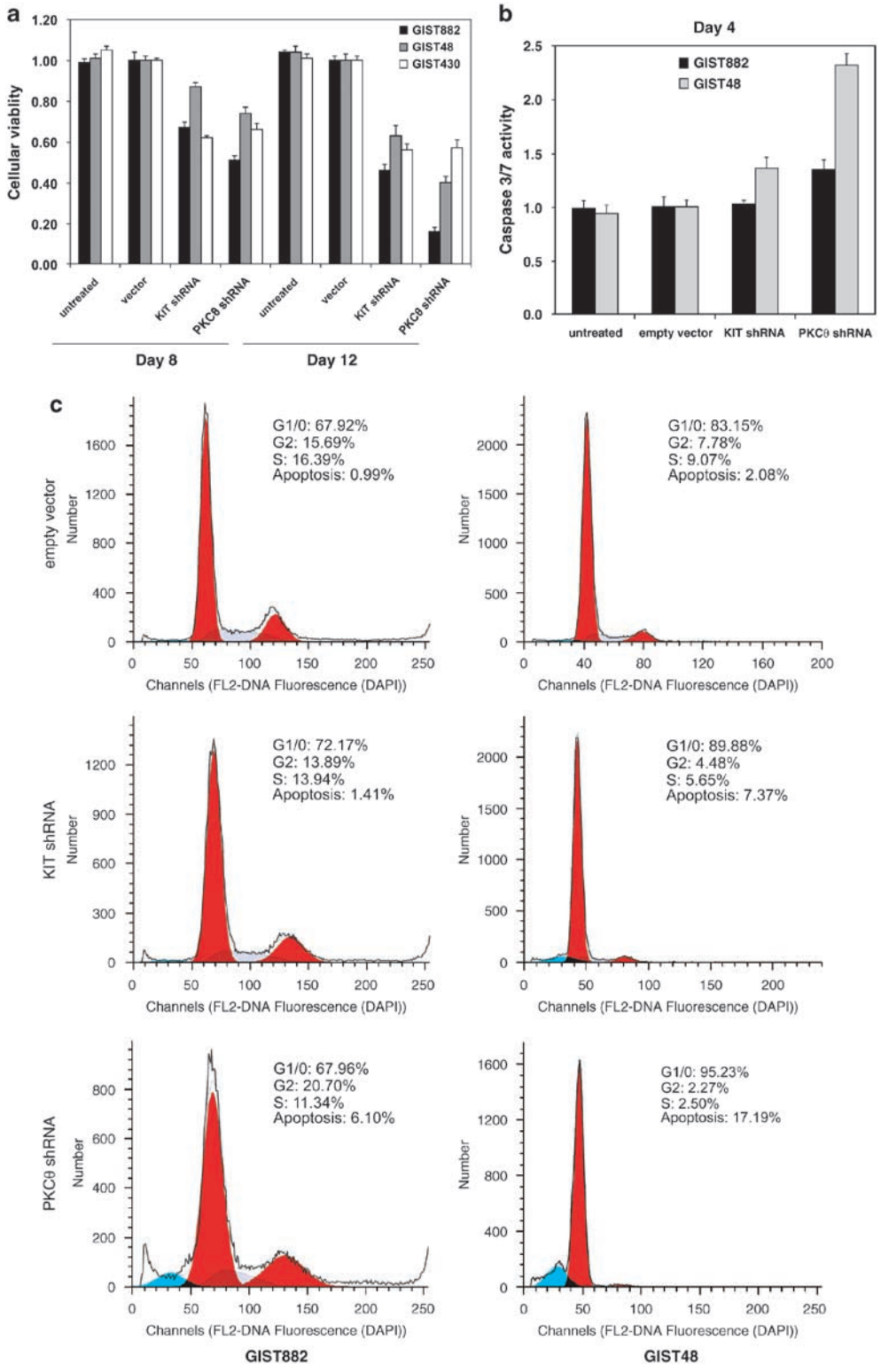

Figure 4 (**a**) Cell viability was evaluated in KIT-positive GIST882 (black bars), GIST48 (gray bars) and GIST430 (white bars) cell lines, at days 8 and 12 after infection with lentiviral *KIT* and protein kinase C-θ (*PKCθ*) short-hairpin RNAs (shRNAs). Viability was analysed using Cell-titer Glo ATP-based luminescence assay. The data were normalized to the empty lentivirus control infections, and represent the mean values (±s.d.) from quadruplicate cultures. (**b**) Apoptosis was evaluated in GIST882 (black bars) and GIST48 (gray bars) cell lines, at day 4 after infection with lentiviral *KIT* and *PKCθ* shRNAs. Caspase 3/7 activity was measured using a Caspase-Glo luminescence assay. The data were normalized to the empty lentivirus control infections, and represent the mean values (±s.d.) from quadruplicate cultures. (**c**) Cell-cycle analyses were performed in GIST882 and GIST48 cells at 8 and 4 days after infection by lentiviral *KIT* or *PKCθ* shRNA constructs, respectively. Apoptotic response was greater, in both cell lines, after treatment with *PKCθ* shRNA than after treatment with *KIT* shRNA.

KIT and PKCθ have highly integrated biologic functions in GIST. In addition, preliminary studies suggest that PDGFRA—an alternate oncoprotein in GIST—is also repressed after PKCθ knockdown (Supplementary Figure 3). These findings highlight that PKCθ pathway therapeutic inhibition warrants clinical evaluation as a novel strategy to downregulate KIT and PDGFRA oncoproteins, including those with imatinib-resistance mutations. PKCθ targeting might also be useful therapeutically in pediatric GISTs which coexpress KIT and PKCθ (Janeway *et al*., 2007), and respond poorly to imatinib (K Janeway, personal communication). Further studies are needed to determine whether PKCθ inactivation by small-molecule kinase inhibitiors can reproduce the spectrum of findings, demonstrated here, that result from downregulation of PKCθ expression in GIST.

It is particularly intriguing that PKCθ regulates KIT expression in GIST, given that PKCθ and KIT have restricted ranges of expression in human cells, and are both coexpressed at unusually high levels in interstitial cells of Cajal, which are non-neoplastic counterparts of GIST (Southwell, 2003). Although the mechanisms by which PKCθ regulates *KIT* gene expression remain to be determined, there is precedent—in T cells—for PKCθ modulation of transcriptional regulators. Namely, PKCθ regulates RXRs in T cells, and the RXRs are key nuclear transcription factors that homodimerize or heterodimerize with other steroid and retinoid receptor family transcription factors (Rastinejad, 2001). T-cell activation signals regulate expression and transactivation of RXRα, and PKCθ participates in these signaling pathways through interactions with calcineurin, resulting in increased RXRE-dependent transcription (Ishaq *et al*., 2002). Further, catalytically inactive PKCθ does not attenuate RXRE-dependent transcription in the T-cell models, and PKCθ cooperates with calcineurin to induce Fas ligand expression during activation-induced T-cell death (Villalba *et al*., 1999).

Previous studies of the GIST882 line showed parallel inactivation of KIT, AKT and MAPK, after treatment with imatinib (Duensing *et al*., 2004b), suggesting that AKT and MAPK were KIT dependent in GISTs. However, our present studies, based on KIT knockdown by shRNA, while confirming that AKT is KIT-dependent, show that MAPK can be KIT independent, in both imatinib-sensitive and -resistant GIST lines (Figures 2c and 3a). These findings suggest that imatinib-dependent MAPK inhibition, in GIST, might depend in part on inactivation of an alternative imatinib target, other than KIT. And these findings are consistent with those reported in EGFR-mutant lung carcinomas and in GISTs, which emphasize that the PI3-K/AKT survival pathway has a crucial role in oncogenic signaling from mutant tyrosine kinase proteins (Sordella *et al*., 2004; Tarn *et al*., 2006). However, substantial MAPK inactivation resulted from PKCθ knockdown in the imatinib-resistant GIST48 and GIST430 lines (Figure 2c), and therefore PKCθ inhibition can inactivate both PI3-K/AKT survival pathways and MAPK-dependent proliferation pathways in GISTs. This might explain why PKCθ knockdown had more impact than KIT knockdown on inhibition of cyclin A expression, and on cell-cycle arrest and apoptosis in imatinib-resistant GIST48 cells (Figures 2c, 4b and c). In addition, the cellular responses seen after PKCθ knockdown in GIST likely depend on dysregulation of pathways beyond those evaluated in the present study. For example, PKCθ mediates activation of the NFκB transcription factor during T-cell activation (Sun *et al*., 2000; Wang *et al*., 2004), and this PKCθ function is performed through interactions with AKT (Bauer *et al*., 2001). Therefore, it will be worthwhile in future studies to determine whether NFκB is a PKCθ effector in GIST.

The protein kinase C family has crucial regulatory roles in cell growth and cell-cycle progression (Frey *et al*., 1997; Ashton *et al*., 1999; Jiang *et al*., 2002; Deeds *et al*., 2003; Cerda *et al*., 2006). Various studies have shown that PKC suppression generally inhibits cell-cycle progression, whereas PKC activation stimulates cell-cycle progression (Frey *et al*., 1997; Deeds *et al*., 2003; Cerda *et al*., 2006). Therefore, we evaluated cell-cycle checkpoints after PKCθ silencing in our GIST models (Figure 2c). *PKCθ* shRNA knockdown resulted in overexpression of the CDK inhibitors p27 and p21, and the p53 cell-cycle checkpoint protein, all of which have tumor suppressor functions in human cancer. These observations suggest that PKCθ regulates cell-cycle checkpoint pathways in GIST, and there is precedent for such cell-cycle control mechanisms in T cells (Deeds *et al*., 2003).

Lentivirus-mediated *KIT* shRNA knockdown resulted in profound antiproliferative and proapoptotic effects in both imatinib-sensitive and -resistant GIST cell lines, and was associated with PI3-K/AKT signaling pathway inhibition. These findings show that KIT activation continues to serve a crucial oncogenic role in some GISTs that develop clinical resistance to imatinib (Heinrich *et al*., 2006). Notably, individual patients can demonstrate heterogeneous imatinib-resistant mechanisms, for example having different *KIT* secondary imatinib-resistant mutations in separate clinically progressing GIST metastases (Debiec-Rychter *et al*., 2005; Heinrich *et al*., 2006). This resistance heterogeneity, with multiple different kinase-resistant mutations in the same patient, poses a challenge to salvage therapy with alternate KIT kinase inhibitors. Specifically, it is unlikely that a given KIT kinase inhibitor will effectively inhibit the myriad different KIT structural oncoprotein variants that can be encountered in a given patient, at time of clinical progression on imatinib therapy. Therefore, the mechanism revealed in this report, whereby PKCθ knockdown inhibits KIT expression, is of substantial clinical relevance, suggesting that therapeutic PKCθ inhibition might repress expression of imatinib-resistant KIT oncoproteins, irrespective of whatever KIT kinase domain mutations are responsible for the imatinib resistance. The concept of PKCθ therapeutic inhibition is all the more appealing in that PKCθ RNAi knockdown was associated with more apoptosis than seen after KIT RNAi knockdown. Most patients with metastatic GIST benefit from

imatinib therapy, having major responses, but nonetheless do not have complete responses. The residual GIST in these treated patients is generally quiescent metabolically, as evidenced by persistent negative PET scans, and cell proliferation arrest. Nonetheless, a subset of the cells survive, and can eventually progress due to imatinib-resistant mutations, suggesting that GIST survival pathways are not fully inhibited by imatinib. The observations reported here suggest that a PKCθ inhibitor might lead to increased GIST apoptosis and thereby maximize the clinical response. In summary, our findings indicate that PKCθ can not only serve as a diagnostic marker in GIST, but perhaps also as a novel therapeutic target of relevance in patients with both imatinib-sensitive and -resistant GIST.

Materials and methods

Antibodies and reagents

Polyclonal antibodies to KIT were from Dako (Carpinteria, CA, USA). Polyclonal antibodies to PKCθ and S6 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies to AKT, and all phospho-antibodies except phospho-KIT and phospho-STAT1 (Zymed Laboratories, South San Francisco, CA, USA), were from Cell Signaling Technologies (Danvers, MA, USA). Polyclonal antibodies to MAPK were from Zymed. Polyclonal antibodies to PI3-K p85 were from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal mouse antibodies were to cyclin A (Novocastra, Newcastle upon Tyne, UK), PCNA and p53 (Santa Cruz Biotechnology), STAT1 and $p21^{Cip1/WAF1}$ (Zymed Laboratories), $p27^{Kip1}$ (BD Transduction Laboratories, San Jose, CA, USA) and actin (Sigma-Aldrich, St Louis, MO, USA). Lipofectamine and plus reagent were from Invitrogen (Carlsbad, CA, USA). Puromycin and polybrene were from Sigma (St Louis, MO, USA).

Cell lines

GIST882 is a human cell line established from an untreated GIST with a primary imatinib-sensitive mutation in *KIT* exon 13 (K642E; Lux *et al*., 2000). GIST48 and GIST430 are human cell lines established from GISTs progressing on imatinib therapy. GIST48 has a homozygous *KIT* exon 11 mutation (V560D) and a heterozygous *KIT* exon 17 mutation (D820A). GIST430 has heterozygous mutations in *KIT* exon 11 (in-frame deletion) and *KIT* exon 13 (V654A). GIST62 was established from an untreated GIST. This cell line, like the primary tumor from which it was established, has a heterozygous *KIT* exon 11 in-frame deletion mutation (resulting in MYEVQWK552-558T), but has essentially undetectable KIT and PKCθ expression. GIST522 was established from a GIST progressing on imatinib therapy, and has a heterozygous *KIT* exon 11 in-frame deletion mutation (resulting in delEVQWK554-558) but, like GIST62, has essentially undetectable KIT and PKCθ expression. GIST62 and GIST522 are highly imatinib resistant (data not shown), and these cell lines serve as negative controls for interventions directed to KIT or PKCθ, in a GIST cell context. EWS502 is an Ewing's tumor cell line expressing wild-type KIT. 293T cells were used to prepare lentiviral constructs.

PKCθ *and* KIT *shRNA lentiviral constructs and preparations*

The pLKO.1puro (7 kb) lentivirus construct contains a U6 promoter and HIV-1 RNA packaging signal with puromycin- and ampicillin-resistant elements cloned 3′ of the human phosphoglycerate kinase (hPGK) promoter. A cpptCTE was inserted 5′ of hPGK promoter. Human *PKCθ* and *KIT* shRNA constructs were generated by ligating the following annealed oligomers into the unique *Age*I and *Eco*RI sites of pLKO.1puro: *PKCθ* forward 5′-CCGGCATCCAAAGC TGCCACAAGTTCTCGAGAACTTGTGGCAGCTTTGG ATGTTTTTG-3′ and *PKCθ* reverse 5′-AATTCAAAAA CATCCAAAGCTGCCACAAGTTGTGGCAGCTTTGGA TG-3′ and *KIT* forward 5′-CCGGCCATAAGGTTTCGT TTCTGTACTCGAGTACAGAAACGAAACCTTATGGTT TTTG-3′ and *KIT* reverse 5′-AATTCAAAAACATAAG GTTTCGTTTCTGTACTCGAGTACAGAAACGAAACCT TATGG-3′.

Lentivirus preparations were produced by co-transfecting pLKO.1puro empty vector with *PKCθ* or *KIT* shRNA, and helper virus packaging plasmids pCMVΔR8.91 and pMD.G (at a 10:10:1 ratio) into 293T cells. Transfections were carried out using lipofectamine and PLUS reagent. Lentiviruses were harvested at 24, 36, 48 and 60 h post-transfection. Viral titers were determined according to a protocol from Invitrogen, in GIST882 cells. Viral preparations were then stored at −80 °C. Two well-validated shRNAs were used each for *KIT* and *PKCθ* knockdown, and these shRNAs were those—screened from a panel of X *KIT* shRNAs and Y *PKCθ* shRNAs—that accomplished the most efficient knockdowns of their intended targets.

Cell culture and virus infection

GIST882 and EWS502 were maintained in RPMI 1640 with 15% fetal bovine serum (FBS) containing penicillin/streptomycin and L-glutamine. GIST48 and GIST430 were maintained in F10 containing 15% FBS, penicillin/streptomycin, L-glutamine, amphotericin, Mitotracker+ and bovine pituitary extract. GIST and EWS502 cells were seeded in six-well plates. Infections were carried out in the presence of 8 μg/ml of polybrene. Cells were lysed for western blot analysis at 96 h post-infection or harvested for cell-cycle analysis. Following transduction, GIST882 cells were selected for stable expression of the shRNAs using 2.5 μg/ml puromycin. Cell culture images were obtained using Spot software (version 3.5.9 for MacOS) and a Nikon Eclipse TE2000-5 microscope.

Western blotting

Whole-cell lysates were prepared in lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories Hercules, CA, USA). Electrophoresis and western blotting were performed as described previously (Rubin *et al*., 2001). Detection was by chemiluminescence (ECL, Amersham Pharmacia Biotechnology), captured using a FUJI LAS1000-plus chemiluminescence imaging system.

Cell viability and apoptosis analyses

GIST882, GIST48 and GIST430 cells were plated at 5000 cells per well in 96-well flat-bottom plates from Falcon (Lincoln, NJ, USA) and cultured in RPMI 1640 or F10 for 2 days before transduction with lentiviral empty vector, *PKCθ* shRNA or *KIT* shRNA. Proliferation and apoptosis studies were performed after 4, 8 and 12 days using the CellTiter-Glo luminescent assay and the Caspase-Glo 3/7 assay kit from Promega (Madison, WI, USA), and were quantitated using a Veritas Microplate Luminometer from Turner Biosystems

(Sunnyvale, CA, USA). All assays were performed in quadruplicate wells, and were averaged from two independent transductions in each cell line.

Cell-cycle analysis

GIST882 and GIST48 cells in six-well plates were trypsinized, centrifuged and washed once with Hank's balanced salt solution at room temperature after infection with lentivirus for 8 and 4 days, respectively. For nuclear staining, a 46-diamidino-2-phenyl indole-containing solution (nuclear isolation and staining solution; NPE Systems, Pembroke Pines, FL, USA) was added to the cells and the cell suspension was immediately analysed in a flow cytometer (NPE Quanta; NPE Systems). Data analysis was performed using Modfit LT software 3.1 (Verity Software House, Topsham, ME, USA).

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